Reference: *Biol. Bull.* **183**: 211–219. (October, 1992)

Cell Cooperation During Host Defense in the Solitary Tunicate *Ciona intestinalis* (L)

VALERIE J. SMITH* AND CLARE M. PEDDIE

School of Biology and Medical Sciences, Gatty Marine Laboratory, University of St Andrews, Fife, KY16 8LB, Scotland

Abstract. Phagocytosis in the solitary ascidian *Ciona intestinalis* was investigated using mixed and separated populations of blood cells *in vitro*. Only the vacuolar and granular amoebocytes were seen to ingest bacteria, and when the serine protease inhibitors, STI, or benzamidine were added to monolayers of mixed cell types, uptake was significantly reduced. Analyses carried out with isolated cells revealed that phagocytosis was enhanced by incubation of the bacteria in blood cell lysate supernatants (CLS) that had been pre-treated with LPS. By contrast, pre-incubation of the bacteria in CLS preparations that were inhibited by benzamidine produced lower levels of phagocytosis. Treatment of the bacteria with plasma also failed to promote uptake, and there was no detectable agglutination of the bacteria by CLS. As lysate supernatants made from morula cells, but not other cell types, were effective in promoting phagocytosis, we propose that opsonins are derived from the morula cells, and that phagocytosis involves cooperation between different cell populations. Moreover, as the morula cells are the principal repositories of prophenoloxidase and an associated serine protease (factors which are activated by LPS but blocked by STI or benzamidine), prophenoloxidase or the protease may be involved in the opsonic phenomenon in a similar way to that previously reported for arthropods.

Introduction

The recognition of non-self is a fundamental aspect of an animal's immune network and a major determinant of the host's ability to survive microbial infection or parasitization. Invertebrates lack specific immunoglobulins and specialized T lymphocytes in their defense repertoires, but are able to mount highly efficient cellular and humoral defense strategies against foreign agents (Ratcliffe *et al.*, 1985; Smith, 1991). An understanding of the biochemical basis for recognition and blood cell activation in these animals is important in tracing the evolution of immune capability and in the identification of suitable non-vertebrate models for experimental study. The deuterostomes, and especially the ascidians, are a particularly significant group because of their phylogenetically strategic position close to the vertebrates (Bone, 1979).

Several workers have examined the cellular responses of ascidians using both *in vivo* (Smith, 1970; Anderson, 1971; Parrinello *et al.*, 1977; Raftos and Cooper, 1991) and *in vitro* methods (Rowley, 1981; 1982a) (see also review by Pestarino, 1991). However, the responses of the cells to foreign onslaught, particularly *in vitro*, have been studied only with mixed blood cell populations. There have been no investigations of separated cells or attempts to assess the extent of interaction between the various cell types.

Although immune reactivity in vertebrates entails cooperation between the different leucocyte groups, the importance of cell-cell cooperation in invertebrate host defense has only been demonstrated for the arthropods (Söderhäll *et al.*, 1986; Persson *et al.*, 1987; Anggraeni and Ratcliffe, 1991). In both crustaceans and insects, the granular-type cells appear to mediate cell cooperation by providing the opsonins necessary for efficient uptake of bacteria by the phagocytes (Söderhäll *et al.*, 1986; Anggraeni and Ratcliffe, 1991). In crayfish, they also donate factors which promote encapsulation (Persson *et al.*, 1987). As yet, the cell signals involved have not been fully characterized, but the prophenoloxidase activating (proPO) system, contained within the granular and se-

Received 4 March 1992; accepted 7 July 1992.

* To whom reprint requests and correspondence should be addressed.

Abbreviations: STI, Soyabean trypsin inhibitor; LPS, Lipopolysaccharide; CLS, whole blood cell lysate supernatant; MAC, marine anticoagulent; MS, marine saline; CAC I, citrated cacodylate buffer; CAC II, cacodylate buffer; proPO, prophenoloxidase; L-dopa L-hydroxyophenylanaline; MLS, morula cell lysate supernatant; CcLS, cell lysate supernatant excluding morula cells; pNA, p-nitroaniline.

migranular cells or t[illegible]rvalent, has been implicated in the phenome[illegible]äll *et al.*, 1986; Anggraeni and Ratcliffe, [illegible]proPO system in crustaceans and insect[illegible]ascade of serine proteases and other fac[illegible]specifically triggered by microbial carboh[illegible]ely lipopolysaccharides (LPS) or β 1,3-glucans [illegible]iews by Söderhäll, 1982; Söderhäll and Smith, 1986a, b; Smith and Söderhäll, 1986; Johansson and Söderhäll, 1989; Smith, 1991). Because phenoloxidase activity occurs exclusively in the granular type cells, it also represents a useful marker for the purity of separated cells in functional evaluations of immune reactivity (Söderhäll and Smith, 1983; Söderhäll *et al.*, 1986; Smith and Söderhäll, 1991; Chisholm and Smith, 1992). So far, few suitable markers have been identified for the cell populations in other invertebrate phyla.

Recently, however, the separation technique devised for crustacean hemocytes has been successfully applied to the solitary ascidian, *Ciona intestinalis* (Smith and Söderhäll, 1991). Five cell bands were obtained, and the enzyme prophenoloxidase was found to reside primarily in the morula cells (Smith and Söderhäll, 1991). Further analyses have established that phenoloxidase activity also occurs in the morula cells of several ascidian species and in individuals of *C. intestinalis*, as in arthropods, it exists as a proenzyme which is activated by proteases or LPS but not other carbohydrates (Jackson *et al.*, in press). Furthermore, the enzyme is blocked by the serine protease inhibitors, STI and benzamidine, (Jackson *et al.*, in press) and seems to be associated with an LPS-sensitive protease contained within the blood cells (Jackson and Smith, unpubl. obs.).

The present investigation was conducted to examine the cellular defenses of urochordates and, by using separated cell populations, to assess the extent of any cell cooperation involved. We chose to examine phagocytosis because it is a universal process which serves in surveillance against invading micro-organisms in nearly all animals. We show that uptake of bacteria is achieved only by the phagocytic amoebocytes and is strongly influenced by factors (opsonins) derived from the morula cells. In addition, by treating the cells or opsonizing preparations with LPS or serine protease inhibitors, we explored the possibility of a link between the opsonic effect and the phenoloxidase and protease activities contained within the morula cells.

Materials and Methods

Animals

Specimens of *C. intestinalis* were collected from the west coast of Scotland and maintained in aerated circulating seawater (32 ± 2‰; 10 ± 2°C) until use. The animals were blotted dry to remove any excess seawater and bled by removal of the tunic and puncture of the pericardium. The blood was drained from the perivisceral and pericardial cavities, care being taken not to contaminate the blood with pyrogens from the gut. The blood (usually 1–2 ml per animal) was diluted 1:1 with ice-cold marine anticoagulant (MAC) (0.1 *M* glucose; 15 m*M* trisodium citrate; 13 m*M* citric acid; 10 m*M* EDTA; 0.45 *M* NaCl; pH 7.0) and processed immediately.

Cell separation

The blood cell populations of *C. intestinalis* were separated by a modification of the density gradient centrifugation method described by Söderhäll and Smith (1983) and Smith and Söderhäll (1991). Briefly, freshly collected blood (approximately 2 ml), diluted 1:1 in MAC was spun through preformed continuous gradients of 60% Percoll (Pharmacia, Uppsala, Sweden) in 3.2% NaCl, at 1900 g for 10 min, at 4°C. The cell types were identified according to the criteria given by Rowley (1981). The phagocytes and morula cells were then further purified by a second centrifugation on preformed gradients of 40% Percoll, while the pigment cells and stem cells were enriched on secondary gradients of 80% Percoll.

The separated cell populations were removed from the gradients and washed once in 10 ml of marine saline (MS) (12 m*M* $CaCl_2 \cdot 6H_2O$; 11 m*M* KCl; 26 m*M* $MgCl_2 \cdot 6H_2O$; 45 m*M* tris; 38 m*M* HCl; 0.45 *M* NaCl; pH 7.4) before resuspension in 800 μl MS. Phenoloxidase and protease activities in the blood cell suspensions were determined for each cell band; L-dopa or S-2337 were used as described below.

Preparation of blood cell monolayers

Blood cell monolayers from individuals of *C. intestinalis* were prepared by a modification of the methods given in Smith and Ratcliffe (1978) and Söderhäll *et al.* (1986). For the mixed cell monolayers, 1.2 ml of freshly collected blood, diluted 1:1 with MAC, was washed once with 5 ml of MS, and resuspended in 1.2 ml MS. Two hundred microlitres of this mixed cell suspension was laid onto each clean, pyrogen-free, glass coverslip in tissue culture grade sterile six-well trays (Sterilin, Feltham, England), and the coverslips were incubated at 20°C for 60 min to allow the cells to settle, attach and spread on the glass surface. Each coverslip was then washed twice with 1 ml of MS and moistened with a fresh 0.5 ml volume of MS.

For separated blood cell monolayers, the cells were removed from the gradients with a pasteur pipette, washed once in MS, and resuspended in fresh MS at a concentration of ca 1×10^8 ml^{-1}. To each coverslip was added 200 μl of the cell suspension and the cells were incubated and washed as above.

Preparation of blood cell lysate supernatants

The diluted blood (about 5 ml) from 4–5 large (7.5–14 cm long) animals was pooled, and the cells were pelleted

by gentle centrifugation (800 g, 10 min, 4°C). The supernatant was discarded, and the cells were washed twice in ice-cold citrated cacodylate buffer (CAC I) (10 m*M* sodium cacodylate, 100 m*M* trisodium citrate, 0.45 *M* NaCl; pH 7.0) before homogenization with a glass piston tissue grinder in citrate-free cacodylate buffer (CAC II) (10 m*M* sodium cacodylate; 50 m*M* $MgCl_2 \cdot 6H_2O$; 50 m*M* $CaCl_2 \cdot 6H_2O$; 0.45 *M* NaCl; pH 7.0). The homogenate was then spun (40,000 g, 20 min, 4°C) and the resulting supernatant, designated CLS, used as enzyme source.

For some phagocytosis experiments (see below), cell lysate supernatants were prepared from separated cells. One, designated MLS, was prepared from the morula cell band, while a second, the control (CcLS), was made from all the cell bands excluding the morula cell band. In both cases, the cell bands were removed from the gradients, pooled where necessary, washed in 10 ml of MAC, and resuspended in 1 ml aliquots of fresh citrated CAC I buffer. The two suspensions were spun at 800 g for 10 min at 4°C, resuspended in 600 μl of CAC II, and homogenized with a glass piston tissue grinder (8 min, 4°C) before centrifugation at 14,500 g for 20 min (4°C). Protein in the CcLS was adjusted to match that of the MLS, and phenoloxidase and protease activities in each of the lysate supernatants were determined as below.

Preparation of plasma

Undiluted blood (approximately 2 ml per animal) was collected from individual medium sized animals (4–7 cm long) and placed into ice-cold polycarbonate tubes. The samples were immediately centrifuged (800 g, 10 min), the supernatants decanted and used as plasma.

Measurement of enzyme activities

Phenoloxidase activity was determined spectrophotometrically at 490 nm after treatment with trypsin or, for controls, CAC II buffer; L-dopa was used as substrate as in Smith and Söderhäll (1983a, 1991). Enzyme activity was expressed as the change in absorbance at 490 nm per min per mg protein.

Protease activity was assayed according to the method of Söderhäll (1983). Briefly, 100 μl of blood cell lysate supernatants of *C. intestinalis* were pre-incubated with an equal volume of lipopolysaccharide (LPS) (from *Escherichia coli* 0111:B4, phenolic extraction), at a concentration of 1 mg ml^{-1} in tris buffer (0.1 *M* tris; 50 m*M* HCl; 10 m*M* $CaCl_2 \cdot 6H_2O$; pH 8.0). For controls, CLS was mixed with 100 μl of tris buffer. After 10 min at 20°C, 200 μl of tris buffer, and 100 μl of the chromogenic substrate, Bz-Ile-Glu (-O-piperidyl) Gly-Arg-pNA · HCl (S-2337) (Kabi-Vitrum, Stockholm, Sweden) (1 mg ml^{-1} in tris buffer) were added to each tube, and the samples were incubated for a further 60 min. The reaction was terminated by the addition of 100 μl of 50% acetic acid, the samples spun at 2500 g (10 min), and the absorbances read against a blank, consisting of buffer, substrate and elicitor, at 405 nm. Enzyme activity was calculated as nmol p-nitroaniline (pNA) released per minute per mg protein.

Protein

Protein in the various cell lysates and plasma was determined by the method of Bradford (1976); bovine serum albumin was used as standard.

Preparation of bacteria

The bacterium, *Psychrobacter immobilis*, formerly *Moraxella sp.* (NCMB 308), was cultured and prepared as described in Smith and Ratcliffe (1978). Washed bacteria were suspended in MS at a concentration of 5×10^8 ml^{-1} and either used immediately or pretreated with various cell lysate supernatants, as described below, before presentation to the cells.

Phagocytosis assays in vitro

The optimal incubation time for bacterial uptake by blood cells of *C. intestinalis* was investigated by challenging monolayers prepared from mixed cells with 100 μl of washed bacteria in MS (*i.e.*, 5×10^7 per monolayer). Controls received 100 μl of MS, and all cultures were incubated on a rocking platform for time intervals ranging from 30 min to 3 h at 20°C. On the basis of this experiment (see Results below), the cell:bacteria cultures in all subsequent experiments were incubated for 2 h.

To examine the effect of serine protease inhibitors on the phagocytic response, monolayers of mixed cells were incubated with either 100 μl of STI in MS (to give a final concentration of 0.143 mg ml^{-1}) or 100 μl of benzamidine (final concentration 14.3 m*M* in MS) before addition of the bacteria. Controls received 100 μl MS, and all cultures, except one of the controls which was given 100 μl of MS, were overlaid with 100 μl of bacteria and incubated as above.

The uptake of bacteria by individual cell types was studied with monolayers prepared from separated cells as described above. To determine whether the uptake of bacteria by isolated phagocytes is influenced by factors present in the cells and entails cell cooperation, monolayers of separated phagocytic amoebocytes were challenged with bacteria pre-incubated in different blood cell lysate supernatants.

Firstly, the bacteria were pre-treated in CLS by incubating 100 μl of washed bacteria (5×10^8 ml^{-1} in CAC II) in 4 ml of CLS (protein concentration of approximately 0.01 mg ml^{-1}). Previously, the CLS had been reacted with 100 μl of LPS (1 mg ml^{-1} in CAC II) (10 min, 20°C).

Further 100 μl samples of the bacteria were incubated in CLS that had been pre-treated with 100 μl CAC II instead of LPS. Second, bacteria were incubated in CLS that had been pre-treated with benzamidine. For this, 4 ml of CLS was mixed with 1 ml of 100 m*M* benzamidine in CAC II (final concentration of 20 m*M*) before addition of LPS and incubation with bacteria as above. Tubes in which CAC II buffer was substituted for the benzamidine were run in parallel as positive controls. Other bacterial samples (100 μl) were incubated in 4 ml of freshly prepared plasma for comparison with the CLS treatments. Finally, bacteria were pre-incubated either in morula cell lysate supernatants (MLS) or in lysate supernatants made from all cell types excluding the morula cells (CcLS). In each case, 500 μl of the respective cell lysate supernatant, pre-treated with 50 μl of LPS, were incubated with 50 μl of bacteria.

All bacteria:lysate, bacteria:buffer, or bacteria:plasma mixtures were incubated for 1 h at 20°C. The bacteria were then washed three times in MS, resuspended in 0.5 ml of fresh MS, and incubated with the phagocytes as above. In every case, controls received bacteria pre-incubated in CAC II buffer only. Protease activities in the lysate supernatants were determined as above. Protease, rather than phenoloxidase, activity was used as a marker for morula cell products because the activity of this enzyme generally shows less variation between animals (data not shown). Cell viability was checked by trypan blue exclusion in all experiments, and bacterial uptake was determined as described below.

Evaluation of bacterial uptake

After incubation, the monolayers were gently washed with three 1 ml changes of MS, fixed in 2 ml of 10% seawater formalin, and observed under phase contrast optics of a Leitz Diaplan microscope. Intracellular bacteria were distinguished from extracellular forms according to the criteria given in Smith and Ratcliffe (1978), and the percentage of blood cells containing one or more ingested bacteria was determined by counting at least 200 cells per coverslip. For each experiment, phagocytosis was determined on duplicate monolayers for each animal, and with the opsonization assays, uptake was assessed for 12 animals using six different lysate supernatants.

Titration of agglutinins

The presence of bacterial agglutinins in blood cells of *C. intestinalis* was investigated as follows. Serial two fold dilutions of 50 μl of CLS in CAC II were made in U-bottomed microtitre trays (Sterilin). Control wells received 50 μl of CAC II instead of the CLS samples, and 50 μl of *P. immobilis* (ca 5×10^8 ml^{-1} in CAC II) was added to every well. The trays were incubated at 20°C for 24 h, and the titers were recorded as the reciprocal of the last CLS dilution showing unequivocal agglutination. The titer was assessed visually and also at 570 nm using an agglutination program on a microplate reader (Dynatech).

Statistical analyses

Differences in levels of uptake of the bacteria by the cells between treatments, and differences in enzyme activities in the cell lysate supernatants between controls and experimentals, were analysed by the Students *t*-test. Differences were considered significant when $P \leq 0.05$.

Results

Cell separation

The blood cells of *C. intestinalis* were separated by density gradient centrifugation, yielding six distinct bands of cells (Table I). Scrutiny of these cells under phase contrast optics revealed eight, morphologically different cell types. They were identified, according to the terminology given by Rowley (1981, 1982a), as signet ring cells (band 1), non-vacuolar hyaline amoebocytes (band 2), vacuolar amoebocytes (band 3), granular amoebocytes (band 3), morula cells (band 4), compartment cells (band 4), pigment cells (band 5), and small, undifferentiated cells (band 6) that resemble vertebrate lymphocytes, and that we designate stem cells because of their similarity to the progenitor cells of other invertebrate species (Table I). We were unable to distinguish a band of cells corresponding to the refractile amoebocytes described by Rowley (1981, 1982a, b), although the reasons for this are unknown.

Only the vacuolar and the granular amoebocytes occupying band 3 were seen to ingest bacteria *in vitro*, so all the cells collected from this band are classified as phagocytic amoebocytes (see below). Likewise, as the morula and compartment cells differed from each other only in terms of their degree of vacuolation, they are referred to collectively as morula cells. The relative proportion of each cell type in whole blood is shown in Table I. These are unlikely to represent absolute values, however, as a number of factors, including body size, season, and allogeneic stimulation, are known to affect differential blood cell counts in ascidians (Smith, 1970; Biggs and Swinehart, 1979; Raftos and Cooper, 1991).

Phenoloxidase and protease activities significantly above control values were found only in the morula cell band ($P = 0.016$ and 0.045, respectively). Slightly enhanced protease activities were detectable in the phagocytic cell band although these were not significantly higher than the controls ($P = 0.461$, Table I). Whether this was a result of contamination by products released by the morula cells during bleeding or separation is not clear. In general, enzyme activities in the morula cell band were approximately three times higher than in the other cell bands or the controls (Table I). Measurement of phe-

noloxidase and protease activities in the signet ring cell band was prevented by low protein yields. Spot assays on this band, performed as described by Söderhäll and Smith (1983), indicated that enzyme activity was not detectable (data not shown).

Phagocytosis by mixed cell monolayers of C. intestinalis in vitro

Cell viability remained at about 96% for 2 h and at over 90% for 3 h under all the experimental conditions (Fig. 1). Uptake of the bacterium *P. immobilis* on mixed cell monolayers increased from about 7% in the first 30 min to a maximum of 36% at 2 h (Fig. 1). Thereafter, uptake declined to about 23% at 3 h (Fig. 1). This perceived drop in phagocytic activity may represent the detachment of phagocytes from the monolayers over prolonged incubation. Accordingly, 2 h was taken to be the optimal incubation time in terms of both cell viability and uptake in all subsequent experiments.

Treatment of the mixed cell monolayers with the serine protease inhibitor, STI, significantly depressed the level of uptake of bacteria from 39 to 16.6% over 2 h ($P = 0.029$) (Table II). A similar effect was observed with benzamidine, where the level of phagocytosis was reduced to 13.4% over the same time period ($P = 0.009$) (Table II). Again, cell viability remained at about 96% or better.

Phagocytosis by separated phagocytes of C. intestinalis in vitro

The *in vitro* phagocytic ability of each of the separated cell types is shown in Table I. Uptake of the bacteria *P.*

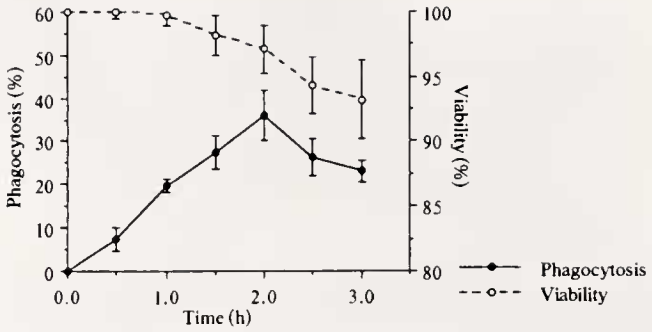

Figure 1. Viability and phagocytosis in mixed blood cell monolayer cultures from *Ciona intestinalis*. The experiments were repeated five times. The values represent mean with bars representing SE. Phagocytosis of the bacteria *Psychrobacter immobilis* was determined under phase contrast optics as described in Materials and Methods. Viability was determined by trypan blue exclusion.

immobilis was achieved only by the phagocytic amoebocytes collected from band 3 of the Percoll gradients. Under these culture conditions, about 5% of the cells were seen to ingest bacteria (Table III), whereas about 39% of the cells were seen to contain intracellular bacteria in the mixed cell cultures (Table II). There did not appear to be preferential phagocytosis either by the non-vacuolar amoebocytes over the granular cells or vice versa (data not shown).

When the phagocytic amoebocytes were challenged with bacteria pre-incubated with LPS-treated CLS, uptake was significantly increased from 5.12 to 42.82%

Table I

Phenoloxidase and protease activities in different blood cell types of Ciona intestinalis

[a] Band	[b] Cell type	[c] Cell count	[d] Phenoloxidase activity (ΔA_{490} min^{-1} mg protein^{-1})	[e] Protease activity (nmol pNA released min^{-1} mg protein^{-1})	[f]n
1	Signet ring cells	*ca.* 9%	NT	NT	6
2	Hyaline leucocytes	*ca.* 16%	0.168 ± 0.045	8.33 ± 3.69	6
3	Phagocytic amoebocytes	*ca.* 37%	0.149 ± 0.052	11.35 ± 2.13	6
4	Morula cells	*ca.* 7%	0.454 ± 0.109*	25.09 ± 4.24*	6
5	Pigment cells	*ca.* 10%	0.130 ± 0.021	7.40 ± 1.49	6
6	Stem cells	*ca.* 19%	0.157 ± 0.025	7.91 ± 3.77	6
—	[g] Control	—	0.148 ± 0.018	6.79 ± 1.34	6

[a] Band on gradients, as counted from the meniscus.

[b] Cell type as identified under phase-contrast optics.

[c] The proportion of each cell type was determined from differential counts of whole blood. A minimum of 600 cells per animal were scored under phase-contrast optics.

[d] The cells from each band were removed from the gradients and pre-incubated with trypsin before the addition of L-dopa. Phenoloxidase activity was then determined spectrophotometrically at 490 nm. NT = not tested.

[e] The cells from each band were removed from the gradients and pre-incubated with LPS before the addition of the chromogenic substrate S-2337. Released p-nitroaniline was determined spectrophotometrically at 405 nm. NT = not tested.

[f]n = number of animals tested.

[g] Controls consisted of cells collected from each cell band pre-incubated with CAC 11 instead of the elicitor before addition of the enzyme substrate. Control values for both enzyme activities were similar for all bands.

* Significantly different compared with control ($P \leq 0.05$).

Table II

Phagocytosis in m[illegible] monolayer cultures from individuals [illegible]

[a] Treatment	[b] Percentage phagocytosis	[c]n
Benzam[illegible] M)	13.48 ± 4.50*	5
STI (0.[illegible]	16.60 ± 2.51*	5
MS (con[illegible]	39.00 ± 4.68	5

[a] Mixed cell monolayers were treated with 100 μl of STI (final concentration 0.143 $mgml^{-1}$) or benzamidine (final concentration 14.3 m*M*), or, for controls, 100 μl MS, before the addition of bacteria.

[b] Proportion of phagocytes containing one or more intracellular bacterium of *P. immobilis*, as determined under phase-contrast optics as in Materials and Methods. Values are means ± SE.

[c] n = number of experimental runs.

* Significantly different compared with control ($P \leq 0.05$).

($P < 0.001$) (Table III). Significantly enhanced uptake was also recorded with the bacteria pre-incubated in CLS without LPS ($P < 0.001$), although this was substantially lower at 20.57% (Table III). Related enzyme assays of the lysates showed that protease activity was significantly higher in the LPS-activated CLS than in the CLS alone ($P = 0.05$). Pre-treatment of the CLS with benzamidine significantly reduced the subsequent uptake to 12.58% ($P = 0.048$), and the enzyme activities in this CLS were similar to those in the CLS alone ($P = 0.871$, Table III). Pre-incubation of the bacteria in plasma also produced no significant increase in uptake over the controls, with only 4.8% of the cells containing one or more bacteria ($P = 0.974$, Table III).

The opsonic effect of the CLS seems to be due, at least in part, to factors contained within the morula cells, since lysate supernatants made from these cells (MLS) were effective in raising the level of phagocytosis from 4.2% in the controls to 26.1% ($P < 0.001$, Table IV). Lysate supernatants made from all the other cell types, excluding the morula cells, (CcLS) were, on the other hand, ineffective in promoting uptake which remained at 8.6%, a value close to that obtained with the controls (Table IV). Again, protease activities in the MLS were significantly higher than in the controls ($P < 0.001$), whereas protease activities in the CcLS were not significantly different to control values ($P = 0.143$). Thus, *in vitro* phagocytosis of bacteria by individuals of *C. intestinalis* appears to entail cooperation between different blood cell types, and the opsonic effect may be linked to the presence of high protease activity in the morula cells.

Titration of agglutinins

Agglutination towards the bacterium *P. immobilis* was not observed in any of the CLS samples, indicating that the opsonic effect seen in the preparations described above was not due to aggregation of the bacteria in the CLS fractions.

Discussion

Previous studies of phagocytosis by urochordate blood cells have used *in vitro* methods with mixed cell cultures. While this approach overcomes some of the complications inherent *in vivo*, the failure to use purified populations of cells may obscure any interactive events between the different cell types. With mixed cell monolayers, Rowley (1981) has reported that phagocytosis in individuals of *C. intestinalis* is carried out by the hyaline and granular amoebocytes. However, he was only able to distinguish phagocytic activity in the vacuolar, as opposed to the non-vacuolar, hyaline amoebocytes through ultrastructural analysis (Rowley, 1982a). In the present study of separated cell populations, we have been able to confirm that uptake is achieved only by the vacuolar hyaline and granular cells (*i.e.*, phagocytic amoebocytes) *in vitro*, and to show that phagocytosis in separated cell cultures is much lower than in mixed cell monolayers. More importantly, we have shown that phagocytic vigor is restored by pre-treating the bacteria with blood cell lysate supernatants. Thus, we have demonstrated for the first time that factors (opsonins) that promote phagocytosis by the amoebocytes reside in the blood cells of *C. intestinalis*.

By pre-treatment of the test bacteria with lysate supernatants of separated cell populations, we have also established that these opsonic factors in *C. intestinalis* appear

Table III

The effect of pre-treatment of Psychrobacter immobilis *on uptake by separated phagocytes from* Ciona intestinalis

[a] Treatment of *P. immobilis*	[b] Percentage phagocytosis	[c] Protease activity (nmol pNA released min^{-1} $mg\ protein^{-1}$)	[d]n
CLS + buffer	20.57 ± 1.13*	7.90 ± 0.48	6
CLS + LPS + buffer	42.82 ± 4.79*	15.45 ± 2.67**	6
CLS + LPS + benzamidine	12.58 ± 1.21*	8.24 ± 1.94	6
Plasma	4.81 ± 2.10	NT	3
Buffer control	5.12 ± 0.49	—	6

[a] Cultures of *P. immobilis* were incubated for 1 h in each of the media, as in Materials and Methods. The buffer was CAC II. After pre-treatment, each bacterial suspension was washed twice in MS and resuspended in fresh MS before the cells were challenged.

[b] Proportion of phagocytes with one or more intracellular bacterium of *P. immobilis*, as determined under phase-contrast optics. Values are means ± SE.

[c] Each CLS was pre-incubated with buffer, LPS or benzamidine (final concentration 20 mM) as appropriate before the addition of the chromogenic substrate S-2337. Released p-nitroaniline was determined spectophotometrically at 405 nm after 60 min. Values are means ± SE. NT = not tested.

[d] n = number of experimental runs.

* Significantly different compared with control ($P \leq 0.05$).

** Significantly different compared with CLS + buffer ($P \leq 0.05$).

Table IV

The effect of pre-treatment of Psychrobacter immobilis *with different cell lysate supernatants on uptake by separated phagocytes from* Ciona intestinalis

[a] Treatment of *P. immobilis*	[b] Percentage phagocytosis	[c] Protease activity (nmol pNA released min^{-1} mg protein $^{-1}$)	[d] n
[e] MLS + LPS	26.08 ± 1.79*	[f] 29.61 ± 1.68**	6
[e] CcLS + LPS	8.62 ± 1.42*	[f] 13.41 ± 1.01	6
Buffer control	4.23 ± 0.52	—	6

[a] Cultures of *P. immobilis* were incubated for 1 h in MLS, CcLS or, for controls, CAC H buffer. They were then washed in MS before addition to the monolayers.

[b] Proportion of phagocytes containing one or more intracellular bacterium of *P. immobilis*, as determined under phase-contrast optics. Values are means ± SE.

[c] Protease activities in the MLS and CcLS were determined spectrophotometrically at 405 nm. Each lysate was incubated with LPS before the addition of the chromogenic substrate S-2337. Released p-nitroaniline was determined spectrophotometrically at 405 nm. Values are means ± SE.

[d] n = number of experimental runs.

[e] MLS = morula cell lysate supernatant; CcLS = cell lysate supernatant from all the cell types excluding the morula cells.

[f] Controls for protease activity comprised CcLS or MLS incubated with buffer instead of LPS before the addition of S-2337. Values were similar for both controls.

* Significantly different compared with buffer incubated bacteria (control) ($P \leq 0.05$).

** Significantly different compared with protease activity in controls (8.12 ± 1.45; mean ± SE; n = 6; $P \leq 0.05$).

to be contributed principally by the morula cells. In other ascidians, the morula cells participate in many phases of the cellular defenses. For example, they migrate to, and break down at sites of tunic injury (Smith, 1970), form multilayered capsules around foreign implants (Anderson, 1971), and infiltrate sites of non-fusion reactions between incompatible colonies (Taneda and Watanabe, 1982). The present paper provides substantial evidence that they play an indirect role in phagocytosis and mediate cell cooperation during host defense responses.

Cooperation between blood cell types occurs during phagocytosis in crustaceans (Söderhäll *et al.*, 1986) and insects (Anggraeni and Ratcliffe, 1991), but equivalent events have not previously been found for deuterostome invertebrates. At present, it is unknown whether similar cooperation takes place in related groups (hemichordates and cephalochordates), or the extent to which it accompanies the other defense processes in ascidians. Certainly, cooperative interaction of the blood cells is an important feature of cellular immunoreactivity in fish and other vertebrates, although it is probably mediated through a different pathway to that in invertebrates.

The enhanced uptake recorded in the present study was probably not due to a 'protein effect', as the protein concentrations of the lysate supernatants were carefully adjusted to comparable levels within each experiment. Moreover, an opsonic effect was not seen after pre-treatment of the bacteria with plasma. Nor can the results be explained by bacterial agglutination, as micro-titer assays failed to show detectable bacterial agglutination in the presence of CLS from *C. intestinalis*.

As inclusion of STI or benzamidine in the mixed cell cultures was found to reduce uptake of bacteria without loss of cell viability, and pre-treatment of the CLS with benzamidine before opsonization of the test bacteria impaired its stimulatory effect, we propose that the enhanced uptake recorded in the present study was mediated through serine protease activity. The occurrence of serine protease activity, stimulated by LPS and reduced by benzamidine or other protease inhibitors, in the blood cells of *C. intestinalis* has been reported by Jackson and Smith (unpub. obs.). Furthermore, it co-exists with prophenoloxidase (Smith and Söderhäll, 1991; Jackson *et al.*, in press) and appears to be involved in the activation of phenoloxidase by LPS (Jackson and Smith, unpub. obs.). In the present study, we clarify that protease and phenoloxidase activities in *C. intestinalis* are located in the morula cells and demonstrate that enhanced phagocytosis *in vitro* correlates with high protease activities in the opsonizing CLS or MLS preparations. Since the bacteria were washed extensively after opsonization before challenging the cells, enhanced uptake of the bacteria incubated in LPS-treated CLS or MLS samples cannot be attributed to a direct action of LPS on the phagocytes. Moreover, LPS-treatment of lysate supernatants (CcLS) made from all the blood cell types except the morula cells only weakly promoted phagocytosis above control levels. We therefore propose that the opsonic effect observed with morula cells of *C. intestinalis* is associated with the activation of prophenoloxidase or protease by LPS. A parallel situation has been described for arthropods (Smith and Söderhäll, 1983a; Ratcliffe *et al.*, 1984; Söderhäll *et al.*, 1986; Anggraeni and Ratcliffe, 1991), but, until the opsonin is purified from individuals of *C. intestinalis*, the precise role of phenoloxidase and protease activities in phagocytosis by this animal remains speculative.

As yet, we are uncertain how the opsonins in *C. intestinalis* may be donated by the morula cells *in vivo*, but it is reasonable to predict that in the mixed cell cultures *in vitro*, the opsonin is generated *in situ* by discharge from the morula cells. In this way, it would be available on the coverslips to coat the test bacteria and promote uptake by the cells. An equivalent process has already been described for arthropods (Smith and Söderhäll, 1983a; Leonard *et al.*, 1985), and LPS-induced exocytosis of prophenoloxidase activating proteins from the granular and semi-granular hemocytes *in vitro* has been observed in crayfish (Smith and Söderhäll, 1983b; Johansson and Söderhäll, 1985). Whether the opsonin in *C. intestinalis*

is exocytosed from [illegible]ula cells in a similar way has not been determi[illegible]ough LPS-stimulated release of proteases from [illegible]cells (of undefined type) has been reported fo[illegible] ascidian *Halocynthia roretzi* by Azumi [illegible] Further work is necessary to clarify the situ[illegible]e solitary ascidian *C. intestinalis*.

Fin[illegible] extent to which phenoloxidase and protease activities are linked to other host defense responses in individuals of *C. intestinalis* is unknown. In crustaceans, the proPO system has been shown to participate in clotting (Söderhäll, 1981), mediate encapsulation (Persson *et al.*, 1987), generate cell adhesion molecules (Johansson and Söderhäll, 1988; Kobayashi *et al.*, 1990), and have antimicrobial activity (Söderhäll and Ajaxon, 1982; Söderhäll and Smith, 1986a). While urochordates share with arthropods a reliance upon such defense strategies for protection against microbial exploitation, more research is necessary to discover the underlying biochemical pathways in deuterostomes.

Acknowledgments

We thank the various sports divers, principally from Cupar Sub-Aqua Club (BSAC 1094) and UMBSM Special Branch (BSAC 902), for their voluntary assistance with the collection of specimens. We are grateful to Dr. Alan Jackson and Mrs. June Chisholm for helpful discussion. The work was supported by grants from SERC, the Nuffield Foundation, and the Royal Society.

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